Communications to the Editor

Triple Helix Formation by Oligonucleotides on DNA Extended to the Physiological pH Range

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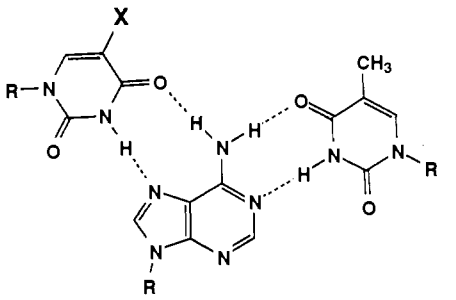
Pyrimidine oligonucleotides recognize extended purine sequences in the major groove of double helical DNA via triple helix formation.[1-3] Specificity is imparted by Hoogsteen base pairing between the pyrimidine oligonucleotide and the purine strand of the Watson-Crick duplex DNA (Figure 1).[1,4] Complexes of triple helical nucleic acids containing cytosine (C) and thymine (T) on the Hoogsteen strand are stable in acidic to neutral solutions *but dissociate on increasing pH*.[1-5] Because oligonucleotide specificity could provide a method for artificial repression of gene expression and viral diseases, it is important to understand those factors controlling triple helix formation in vivo where temporal and spatial intracellular pH (7.0-7.4) is strictly regulated.[6]

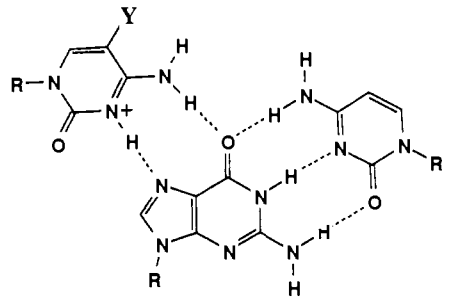
We report here that oligodeoxyribonucleotides which contain 5-bromouracil (Br^5U) and 5-methylcytosine (m^5C) bind duplex DNA at the same homopurine target sequence as their T/C analogues but with greater affinities and over an extended pH range. Oligonucleotides containing uracil (U) bind with lower affinity (Figure 1).

Six oligonucleotides containing combinations of U/C (**1**), U/m^5C (**2**), T/C (**3**), T/m^5C (**4**), Br^5U/C (**5**), and Br^5U/m^5C (**6**) were synthesized by automated methods with thymidine-EDTA (T*) at the 5′ end (Figure 2).[7] The efficiency of double strand cleavage of DNA by oligonucleotide-EDTA·Fe(II) **1-6** was analyzed over the pH range 6.6-7.8 at 25 °C on a plasmid DNA (4.06 kilobase pairs) containing the 15 base pair homopurine target sequence, 5′-$A_5(GA)_5$-3′[1] (Figure 2). The DNA cleavage products were separated by agarose gel electrophoresis and quantitated by scintillation counting of each band (Figure 3).[1a]

Oligonucleotide-EDTA·Fe **1-6** cleave double-stranded DNA at a single site corresponding to the target sequence (Figure 2).



X^5UAT base triplet



Y^5C^+GC base triplet

Figure 1. Isomorphous base triplets formed by incorporation of a third strand in the major groove of double helical DNA parallel to the Watson-Crick purine strand via Hoogsteen base pairing. Substituents (X = Br and Me, Y = Me) at the pyrimidine 5 position protrude from the major groove.

Table I. The Absolute Cleavage Efficiencies of Oligo **1-6**[a]

			cleavage efficiency, pH			
oligo (base)	X	Y	6.6	7.0	7.4	7.8
1 (U, C)	H	H	+	+	-	-
2 (U, m^5C)	H	Me	++	++	+	-
3 (T, C)	Me	H	++	++	-	-
4 (T, m^5C)	Me	Me	++	+++	+	-
5 (Br^5U, C)	Br	H	+++	+++	+	-
6 (Br^5U, m^5C)	Br	Me	++++	++++	+++	+

[a] These were determined by scintillation counting of bands cut from dried gels and are as follows: + = 2-4%, ++ = 5-7%, +++ = 8-10%, ++++ = 10-16%.

The cleavage efficiency of **3** containing C and T decreases sharply above pH 7.0.[1,8] Replacement of C with m^5C (**2**, **4**, and **6**) increases the oligonucleotide affinity and extends the pH range for binding. Substitution of Br^5U for T (**5**) increases binding affinity but does not change the pH profile greatly. Incorporation of both m^5C and Br^5U (**6**) results in a large *increase in cleavage efficiency over an extended pH range*. Oligonucleotides **1** and **2**, constructed with U/C and U/m^5C, show lower binding affinities (Table I).

Substitution at position 5 of pyrimidines could alter the hydrophobic driving force, base stacking, and the electronic complementarity of the Hoogsteen pyrimidine-purine base pairing for triple strand formation. There are two opposing electronic

(1) (a) Moser, H.; Dervan, P. B. *Science* **1987**, *238*, 645. (b) Strobel, S. A.; Moser, H. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7977.

(2) For α-anomers of oligonucleotides, see: (a) Doan, T. L.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J.-L.; Thuong, N. T.; Lhomme, J.; Helene, C. *Nucl. Acids Res.* **1987**, *15*, 7749. (b) Praseuth, D.; Perrouault, L.; Doan, T. L.; Chassignol, M.; Thuong, N.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349.

(3) Lyamichev, V. I.; Mirkin, S. M.; Frank-Kamenetskii, M. D.; Cantor, C. R. *Nucl. Acids Res.* **1988**, *16*, 2165.

(4) (a) Felsenfeld, G.; Davies, D. R.; Rich, A. *J. Am. Chem. Soc.* **1957**, *79*, 2023. (b) Michelson, A. M.; Massoulié, J.; Guschlbauer, W. *Prog. Nucleic Acids Res. Mol. Biol.* **1967**, *6*, 83. (c) Felsenfeld, G.; Miles, H. T. *Annu. Rev. Biochem.* **1967**, *36*, 407. (d) Lipsett, M. N. *Biochem. Biophys. Res. Commun.* **1963**, *11*, 224. (e) Lipsett, M. N. *J. Biol. Chem.* **1964**, *239*, 1256. (f) Howard, F. B.; Frazier, J.; Lipsett, M. N. *Biochem. Biophys. Res. Commun.* **1964**, *17*, 93. (g) Miller, J. H.; Sobell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *55*, 1201. (h) Morgan, A. R.; Wells, R. D. *J. Mol. Biol.* **1968**, *37*, 63. (i) Lee, J. S.; Johnson, D. A.; Morgan, A. R. *Nucl. Acids Res.* **1979**, *6*, 3073.

(5) *Polynucleotides* which contain 5-methylcytosine (m^5C) form triplexes (m^5C^+·G·m^5C triplet) up to pH 8. Lee, J. S.; Woodsworth, M. L.; Latimer, L. J. P.; Morgan, A. R. *Nucl. Acids Res.* **1984**, *12*, 6603.

(6) For discussion of the regulation of intracellular pH in eukaryotic cells, see: (a) Madshus, I. H. *Biochem J.* **1988**, *250*, 1-8. (b) Bright, G. R.; Fisher, G. W.; Rogowska, J.; Taylor, D. L. *J. Cell Biol.* **1987**, *104*, 1019. (c) Boron, W. F. *J. Membr. Biol.* **1983**, *72*, 1-16. (d) Busa, W. B. *Ann. Rev. Physiol.* **1986**, *48*, 389-402. (e) Busa, W. B.; Nuccitelli, R. *Am. J. Physiol.* **1984**, *264*, R409-R438.

(7) Dreyer, J. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 968.

(8) Since the ability of the EDTA·Fe(II) moiety to cleave DNA in Tris buffer increases from pH 6.6 to 7.4,[9] the decrease in cleavage efficiency observed is likely due to a decrease in the binding affinity of the oligonucleotide for its target sequence.

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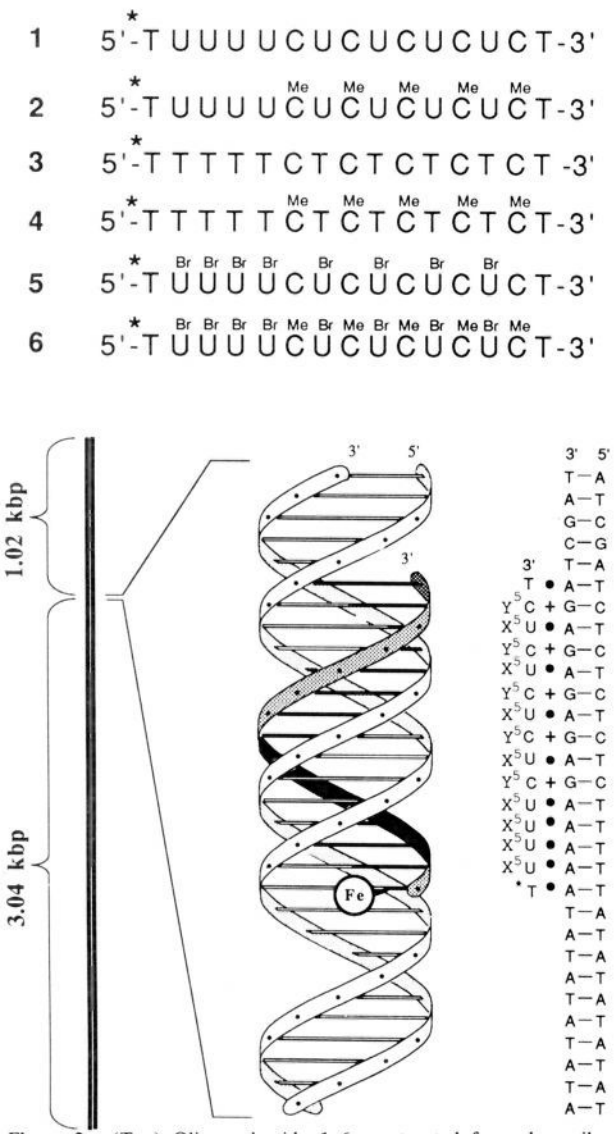

Figure 2. (Top) Oligonucleotide **1–6** constructed from deoxyribonucleotide phosphoramidites containing cytosine (C), 5-methylcytosine (m^5C), uridine (U), thymidine (T), 5-bromouridine (Br^5U), and thymidine-EDTA (T*).[7] (Bottom) Coarse resolution pattern for cleavage of plasmid DNA (4.06 kbp) by oligonucleotide-EDTA **1–6** with simplified model of triple helical complex between the Hoogsteen-bound oligonucleotide (X^5U/Y^5C) and a 15 basepair single site.

effects to consider for Hoogsteen hydrogen bonding between X^5U and A. The electron-withdrawing bromo substituent increases the acidity at N3H (better hydrogen donor) and decreases the electron-donating properties of the carbonyl lone pair (poorer hydrogen acceptor).[10] The electron-donating methyl substituent would have the opposite effect. Methylation of C could stabilize protonation at N3 in the triplex although this stabilization is modest in the uncomplexed C.[10]

We find that the relative stabilities of base triplets are Br^5U·AT>T·AT>U·AT and m^5C^+·GC>C^+·GC (Table I). Oligonucleotides **2** and **3**, which involve the triplet changes m^5C^+·GC/U·AT to C^+·GC/T·AT, show approximately equal binding affinities at or below pH 7.0. This suggests that methylation results in increased binding affinity *irrespective of the methylated pyrimidine*. One possible interpretation is that substitution of methyl for hydrogen at position 5 promotes binding of the oligonucleotide via a hydrophobic effect. In addition, methylation at C causes extension of the pH range (~0.4 units) for triple helix stability. This might be explained by an increase in the pK_a of $N3H^+$ in the triplex. Enhancement of binding by bromo substitution could be due to increased acidity at N3 or enhanced hydrophobicity or both. In the case of mixed oligonucleotides containing Br^5U and m^5C (**6**) it is not inconceivable that a "hydrophobic spine" is created by the Br and methyl moieties aligned on the Hoogsteen strand in the major groove of DNA.

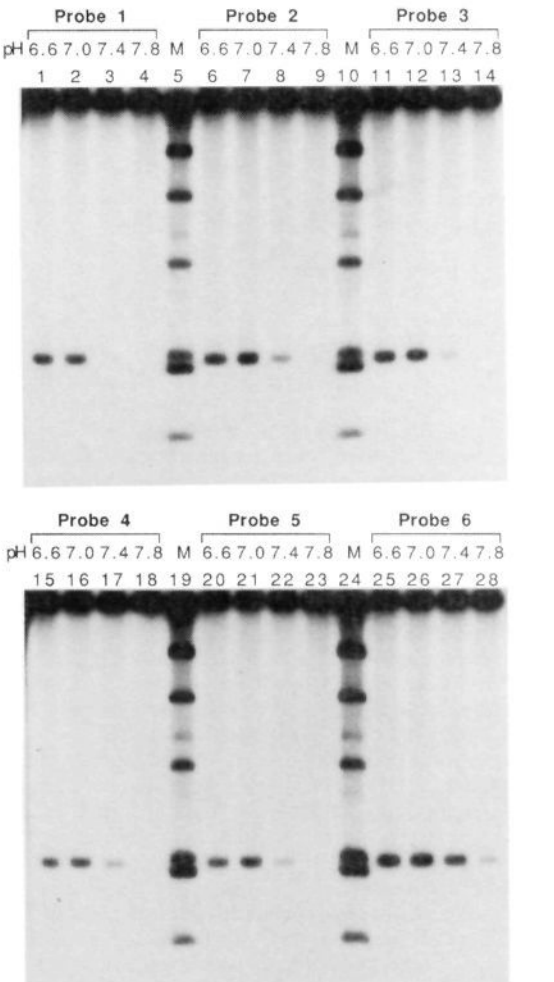

Figure 3. Double strand cleavage of plasmid DNA analyzed on a 0.9% agarose gel. Plasmid pDMAG10 was linearized with Sty I and labeled with [α-^{32}P] TTP, producing a 4.06 kb restriction fragment specifically labeled 1.02 kb from the target sequence.[1a] The ^{32}P-end-labeled DNA was dissolved in buffer containing NaCl, Tris, and spermine and was mixed with oligonucleotide-EDTA **1–6** previously equilibrated for 60 s with 1.5 equiv of Fe(II). After incubation at 0 °C for 60 min, reactions were initiated by the addition of sodium ascorbate (final concentrations: 25 mM Tris·acetate, 1 mM spermine, 100 mM NaCl, 0.8 μM oligonucleotide-EDTA·Fe(II), and 1 mM ascorbate). The cleavage reactions were allowed to proceed for 6 h at 24 °C. The reactions were loaded directly onto an 0.9% agarose gel and electrophoresed at 120 V for 3 h. Cleavage conditions: lanes 1–4, **1**·Fe(II); lanes 6–9, **2**·Fe(II); lanes 11–14, **3**·Fe(II); lanes 15–18, **4**·Fe(II); lanes 20–23, **5**·Fe(II); lanes 25–28; **6**·Fe(II); lanes 1, 6, 11, 15, 20, and 25 are at pH 6.6; lanes 2, 7, 12, 16, 21, and 26 are at pH 7.0; lanes 3, 8, 13, 17, 22, and 27 are at pH 7.4; lanes 4, 9, 14, 18, 23, and 28 are at pH 7.8. Lanes 5, 10, 19, and 24 are DNA size markers obtained by digestion of Sty I linearized pDMAG10 with Eco RI, Pvu I, Bam HI, and Xmn I, resulting in fragments 4058 (undigested DNA), 3067, 2994, 2371, 1687, 1462, 1064, 991, and 664 bp in length.

(9) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934.

(10) pK_a values: deoxyuridine, thymidine, and 5-bromodeoxyuridine are 9.3, 9.8,[11a] and 8.1:[11b] deoxycytidine and 5-methyldeoxycytidine are 4.3 and 4.4, respectively.[11a]

(11) (a) *Handbook of Biochemistry*; 2nd ed.; Sober, H. A., Ed.; CRC Press: Cleveland, OH, 1970. (b) Lawley, P. D.; Brookes, P. *J. Mol. Biol.* **1962**, *4*, 216.

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Alkyne Addition Reactions on Pentaammineosmium(II): The Formation of π-Enol and π-Vinyl Ether Complexes

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The hydration of unactivated alkynes represents an important method of functionalizing this plentiful hydrocarbon resource and has found considerable synthetic use.[1] Transition metals are widely used to catalyze this process as well as the analogous reaction in which alcohols are added across the triple bond.[2] Though π-vinyl ether[3] and π-vinyl alcohol[3,4] complexes are undoubtedly intermediates in these reactions, to our knowledge there have been no reports of such species resulting from an η^2-coordinated alkyne. In an early paper on the reactivity of η^2-alkyne complexes of platinum(II), Chisholm and Clark suggested that addition of methanol occurred across the alkyne bond to produce a vinyl ether intermediate, but this suggestion was later withdrawn.[5] Here we report that the alkyne complex $[Os(NH_3)_5(\eta^2\text{-}CH_3CCCH_3)]^{2+}$ reacts quantitatively with methanol or water to form π-vinyl ether and π-vinyl alcohol complexes, respectively.

Reduction of the precursor $Os(NH_3)_5(OTf)_3$ (OTf = $CF_3SO_3^-$) in the presence of 2-butyne results in a complex, **1**, which is readily characterized as $[Os(NH_3)_5(\eta^2\text{-}CH_3CCCH_3)](OTf)_2$.[6] Though the thermal instability of this material has precluded a successful microanalysis,[7] convincing evidence for this assignment is provided by IR, ^{1}H NMR, and cyclic voltammetric data.[8]

When a methanol solution of the alkyne product **1** is allowed to stand overnight, a new material,[9] **2a**, is isolated which is characterized as the π-vinyl ether containing cation $[Os(NH_3)_5(\eta^2\text{-}cis\text{-}CH_3CH{=}C(OCH_3)(CH_3))]^{2+}$. In addition to ammine resonances, ^{1}H NMR data reveal peaks with chemical shifts and splitting patterns similar to those reported for the free ligand *cis*-2-methoxy-2-butene,[10] and electrochemical measurements provide an $E_{1/2}$ (0.53 V) similar to that reported for other olefin-pentaammineosmium(II) complexes.

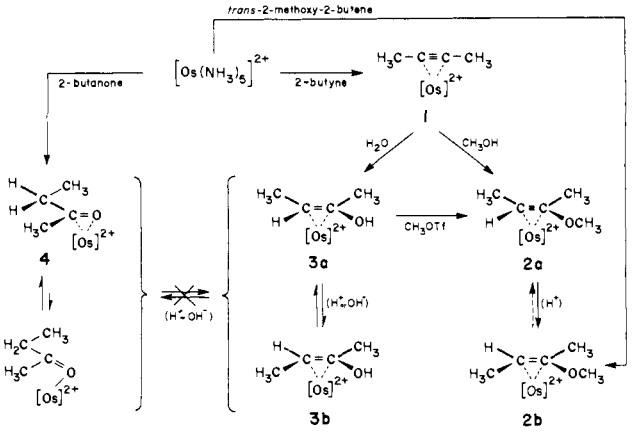

Figure 1. Chemistry associated with π-vinyl alcohol and ether complexes of pentaammineosmium(II).

An aqueous solution of the alkyne product **1** after 8 h yields a new material,[11] **3a**, whose ^{1}H NMR closely resembles that of the vinyl ether **2a**, less the methoxy resonance. In its place is a resonance at 5.00 ppm which is ascribed to the hydroxy proton of the enol cation $[Os(NH_3)_5(\eta^2\text{-}cis\text{-}CH_3CH{=}C(OH)(CH_3))]^{2+}$. Cyclic voltammetric data are consistent with a π-olefin complex showing $E_{1/2}$ = 0.37 V.[12] The infrared spectrum of **3a** as a glaze on a NaCl salt plate features a high frequency absorption at 3475 cm^{-1} which is absent in the IR of a sample of **2a** prepared in similar fashion. This feature is assigned to the enol ν(O-H). The reaction of **1** with water is significantly catalyzed by acid; in a 1 M solution of DOTf the half-life for hydration in aqueous solution is reduced from hours to seconds or less.[13] In the presence of base, an aqueous solution of **1** appears unaltered after 1 h.

Over a period of several days, ^{1}H NMR spectra of an acetone-d_6 solution of **3a** reveal that this complex is unstable with respect to its stereoisomer $[Os(NH_3)_5(\eta^2\text{-}trans\text{-}CH_3CH{=}C(OH)(CH_3))]^{2+}$ (**3b**).[14] The resonances ascribed to the trans isomer are similar to those of the cis form with the exception of the vinyl proton, which manifests a multiplet rather than a pure quartet. A similar discrepancy is found in the comparison of stereoisomers for the free ligand 2-methoxy-2-butene.[10] In acetone, methanol, or water, an equilibrium is reached between **3a** and **3b** in which the trans form (**3b**) is slightly favored ($K_{eq} \cong 1.5$). The addition of either base or acid significantly catalyzes this isomerization.[15]

The ligand *trans*-2-methoxy-2-butene was prepared from *trans*-2-butene following a modification of the procedure reported by Stang et al.[16] By the use of established synthetic procedures,[17] pentaammineosmium(II) was generated in the presence of this alkene resulting in the diamagnetic complex, **2b**. Microanalytical

(1) Hudrlik, P. F.; Hudrlik, A. M. *The Chemistry of the Carbon–Carbon Triple Bond*; John Wiley and Sons: New York; 1978; Part 1.
(2) Utimoto, K. *Pure Appl. Chem.* **1983**, *55*, 1845.
(3) Cutler, A.; Rosenblum, M. *J. Organomet. Chem.* **1974**, *77*, 381.
(4) Hillis, J.; Francis, J.; Ori, M.; Tsutsui, M. *J. Am. Chem. Soc.* **1974**, 4800.
(5) Chisholm, M. H.; Clark, H. C. *Inorg. Chem.* **1971**, *10*, 2557.
(6) (All reactions under anaerobic conditions.) Synthesis of $[Os(NH_3)_5(\eta^2\text{-}CH_3CCCH_3)](OTf)_2$: A solution of $Os(NH_3)_5(OTf)_3$ (800 mg), *N,N*-(DMA) (1.0 mL), 1,2-dimethoxyethane (DME) (10 mL), and 2-butyne (1.0 mL) is stirred with activated magnesium (1 g, turnings; surface cleaned with I_2) for 35 min. The solution is filtered and treated with ether (200 mL). The resulting ppt is collected, washed with ether, and dried under vacuum.
(7) The solid **1** has a half-life of approximately 1 week at 25 °C in the absence of oxygen.
(8) Recorded under anaerobic conditions: ^{1}H NMR (acetone-d_6) 4.82 (b, 3 H), 3.70 (b, 12 H), 2.07 (s, 6 H); CV (acetone; NaOTf) $E_{1/2}$ = −0.10 V, NHE; IR (acetone glaze on NaCl salt plate) ν(C≡C) = 1943 cm^{-1}.
(9) Synthesis of **2a**: 200 mg of **1** are dissolved in 2 mL of MeOH for a period of 18 h. The addition of ether to this solution results in a ppt which is collected and washed with ether. The crude product is purified on column of SP Sephadex C-25 resin by eluting with 0.2 M NaCl and is isolated as the BPh_4 salt. ^{1}H NMR (acetone-d_6, BPh_4^- salt) 1.32 (d, 3 H, CCH_3), 1.63 (s, 3 H, CCH_3), 3.73 (q, 1 H, CH), 3.50 (s, 3 H, OCH_3), 3.69 (b, 12 H), 4.80 (b, 3 H), (BPh_4^-: 6.77 (8 H), 6.92 (16 H), 7.33 (16 H)); ^{13}C NMR (acetone-d_6; OTf^- salt; proton decoupled) 14.4, 58.6, 92.7, 39.9, 15.6 ppm; OTf 121.7 (q); CV (acetone; TBAH) $E_{1/2}$ = 0.53 V, NHE. Anal. Calcd for $C_{53}H_{65}OsON_5B_2$: C, 63.66; H, 6.55; N, 7.00. Found: C, 63.81; H, 6.48; N, 7.09.
(10) Stang, P. J.; Mangum, M. G. *J. Am. Chem. Soc.* **1975**, *97*, 1459.
(11) Synthesis of **3a**: 250 mg of **1** are dissolved in water for 8 h. The crude product is purified on column of SP Sephadex C-25 resin by eluting with 0.2 M NaCl and is isolated as the BPh_4 salt. ^{1}H NMR (acetone-d_6, BPh_4^- salt) 1.27 (d, 3 H, C-CH_3), 1.67 (s, 3 H, C-CH_3), 3.51 (q, 1 H, CH), 5.00 (s, 1 H, OH), 3.72 (b, 12 H), 4.73 (b, 3 H), (BPh_4^-: 6.77 (8 H), 6.92 (16 H), 7.33 (16 H)); CV (acetone, TBAH) $E_{1/2}$ = 0.37 V, NHE; IR (acetonitrile glaze on a NaCl salt plate) 3475 cm^{-1}. Anal. Calcd for $C_{52}H_{63}OsON_5B_2$: C, 63.35; H, 6.44; N, 7.10. Found: C, 62.82; H, 6.31; N, 7.04.
(12) Repeated cycling reveals the partial decomposition of the osmium(III) species; a new species appears with $E_{1/2}$ = 0.49 V, NHE.
(13) After 5 min in a 1 M DOTf/D_2O solution, ^{1}H NMR reveals complete conversion of **1** to a mixture of **2a** and **2b**. Deuterium exchange has occurred at the C1 position.
(14) Characterization of **3b**: ^{1}H NMR (acetone-d_6, BPh_4^- salt) 1.31 (d, 3 H, CCH_3), 1.63 (s, 3 H, C-CH_3), 3.26 (m, 1 H, CH), 5.03 (s, 1 H, OH), 3.72 (b, 12 H), 4.69 (b, 3 H), (BPh_4^-: 6.77 (8 H), 6.92 (16 H), 7.33 (16 H)); CV (acetone, TBAH) $E_{1/2}$ = 0.37 V, NHE.
(15) The addition of Proton Sponge in acetone or NaOMe in MeOH significantly increases the rate of isomerization of **3a**. (In water, both H^+ or OH^- catalyze this process.)
(16) *N*-bromosuccinamide was substituted for *N*-bromoacetamide. NMR of *trans*-2-methoxy-2-butene (CD_3CN) 1.46 (d of q, 3 H), 1.76 (m, 3 H), 3.51 (s, 3 H), 4.42 (q of q, 1 H); GS-MS *m/z* = 86 (108), 85 (41), 71 (105), 55 (101) (see ref 10).
(17) Harman, W. D.; Taube, H. *Inorg. Chem.* **1987**, *26*, 2917.

0002-7863/89/1511-3061$01.50/0